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Factors governing performance of continuous fungal reactor during non-sterile operation - The case of a membrane bioreactor treating textile wastewater

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Factors governing performance of continuous fungal reactor during non-sterile operation—the case of a membrane bioreactor treating textile wastewater

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Abstract White-rot fungi, unlike bacteria in conventional activated sludge system, can degrade wide varieties of textile dyes. Their large scale implementation, however, has been impeded due to lack of appropriate reactor system that can sustain stable performance under non-sterile environment. In this study, contrary to virtually complete decoloration of an azo dye (Acid Orange II, 100 mg L^{-1}) in pure culture batch test, a fungal membrane bioreactor (MBR) achieved 93% removal during long-term non-sterile operation at a hydraulic retention time (HRT) of 1 d. Through a set of novel observations made in MBR and parallel batch tests, the interrelated factors responsible for incomplete dye removal, namely, bacterial disruption, fungal morphology and enzyme washout were identified. As compared to the activity of pure fungus culture, the bacteria-contaminated disintegrated MBR-sludge demonstrated low decoloration and undetectable enzymatic activity, indicating detrimental effect of bacterial contamination. Additional observations suggested close relationship between fungal morphology and enzymatic/decoloration activity under non-sterile environment. This study also demonstrated the occurrence of enzyme washout from MBR and its HRT-specific detrimental influence on removal performance. Based on the observations, certain ways to enhance decoloration were proposed.

Keywords: Decoloration; Membrane Bioreactor (MBR); Non-sterile operation; Dye; White-rot fungi

1. Introduction

Virtually all the known physico-chemical and biological techniques have been explored for treatment of extremely recalcitrant textile dye wastewater; none, however, has emerged as a panacea (Hai et al., 2007). Residual dyes along with other auxiliary chemical reagents used for textile processing impose massive load on wastewater treatment systems, eventually leading to poor color and COD removal performances. The presence of even trace concentration of dyes in effluent is highly visible and the release of such colored wastewater in the ecosystem is a remarkable source of esthetic pollution, eutrophication and perturbations (due to toxicity and persistence) in aquatic life (Robinson et al., 2001).

Biodegradation is an environmentally friendly and cost competitive alternative. However, the conventional aerobic decoloration processes have been proved rather ineffective, while highly toxic aromatic amines are formed during anaerobic azo dye decoloration (Van der Zee and Villaverde, 2005). In this context it is interesting to note that unlike bacterial activated sludge process, aerobic white-rot fungi can degrade wide varieties of recalcitrant compounds including textile dyes (Wesenberg et al., 2003).

The literature is replete with reports demonstrating the excellent capacity of white-rot fungi to degrade recalcitrant dyes in small-scale, sterile batch tests. However, fewer studies have been conducted in continuous reactors, while only a handful of studies involved

investigations under non-sterile conditions (Yang and Yu, 1996; Leidig et al., 1999; Libra et al., 2003; Hai et al., 2006; Blanquez et al., 2008). Deteriorated decoloration is usually reported in the continuous reactors. The typical slow rate of degradation necessitates long hydraulic retention time (HRT) to maintain good level of decoloration during continuous operation (Moreira et al., 1998). In addition, significantly deteriorated performance is observed owing to bacterial contamination (Libra et al., 2003). Regrettably, the available reports only demonstrate the deteriorated fungal decoloration in the continuous reactors without clarifying the factors responsible for such deterioration. A few studies alluded to the factors like bacterial destabilization of fungal activity (Libra et al., 2003) or washout of fungal enzyme from continuous reactors (Zhang and Yu, 2000), but failed to offer any comprehensive picture.

On the other hand, to date, only two studies have reported long-term stable decoloration under non-sterile environment. Hai et al. (2006) developed a membrane-coupled fungi reactor and demonstrated excellent (99%) long-term (50 d) combined (fungi + membrane) decoloration of a selected dye. However, they did not test the efficiency of the system with other structurally different dyes and, also, did not directly address the factors governing fungal decoloration. In another study, Blanquez et al. (2008), by combining the strategies of nutrient-limited condition and partial biomass renovation, demonstrated long-term (110 d) stable decoloration (78%) of a different dye by an air-pulsed bed bioreactor. Then again, their strategy failed to yield good decoloration when exposed to a different textile wastewater under higher level of bacterial contamination. It is obvious that systematic investigation about the factors governing fungal decoloration in continuous reactors under non-sterile environment is the key to formulation of an efficient reactor system.

The objective of this study was, therefore, to conduct in-depth investigations to point out some of the key factors governing fungal decoloration in continuous reactors under non-sterile environment and, based on that, provide insight into enhancement of decoloration activity. Long-term performance of membrane-coupled fungi reactors under different HRT and feeding mode (continuous or fed-batch) was observed. Batch tests with pure fungus culture and reactor-sludge were conducted to accumulate additional supporting evidence. The investigations as a whole enabled identification of the governing factors, their mode of influence and probable interrelations. In the light of that, certain means to enhance decoloration were also proposed.

2. Materials and methods

2.1 Microorganism, dye and synthetic wastewater

The white-rot fungus *Coriolus versicolor*, NBRC 9791 obtained from the NITE Biological Resource Center, Japan was used for this study. A nutrient-sufficient synthetic wastewater was prepared by adding dye (Acid Orange II, 100 mg L^{-1}) and starch (2 g L^{-1})—two common components in real textile wastewater—along with urea (0.1 g L^{-1}) and other nutrients (Hai et al., 2008) into tap water. A significant portion of the poorly soluble starch in the wastewater remained in suspended form. During batch tests, Milli Q water instead of tap water and higher concentrations of starch (4.5 g L^{-1}) and urea (0.4g L^{-1}) were used. Also, during batch tests starch was in soluble form as the final solution was autoclaved. Acid Orange II is a water-soluble, low molecular weight (350), mono-azo dye (Fig. 1a) which provides orange color (peak absorbance: 481 nm).

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2.2 Description and feeding mode of the bioreactors

Based on good performance with a wastewater containing a different dye (Hai et al., 2006), a membrane bioreactor (MBR) was chosen in this study. The working volume of the cylindrical, PVC bioreactor (Fig. 1b) that was used for the first part of this study was 11.8 L. A previously developed (Hai et al., 2008), spacer-filled compact hollow-fiber membrane module (height = 22 cm, diameter = 4.5 cm) was submerged into the reactor. The module contained micro-porous (0.4 μm), hydrophilically treated, polyethylene hollow-fibers with a total surface area of 1.07 m^2. Effluent was filtered out through the membrane by a suction pump with a 5 min on/off cycle (average flux of 0.011 $m^3 m^{-2} d^{-1}$) resulting in an HRT of 1 d. The membrane was periodically backwashed with NaOCl solution (250 mg Cl_2 L^{-1}). The cleaning protocol has been detailed elsewhere (Hai et al., 2008). A diffuser supplied continuous air from the bottom of the reactor with an intensity of 5 L min^{-1} for complete mixing and supply of dissolved oxygen to the microbes. Concentrated synthetic wastewater was diluted with tap water and then supplied into the reactor by pumps controlled by a water level controller. The temperature of the reactor was controlled at 29±1°C. The system was first inoculated with 2.5 g *C. versicolor* (dry wt.) aseptically grown for two weeks in 1 L Erlenmeyer flasks each containing 500 mL of the synthetic media (Sec. 2.1). The reactor was then kept under aeration for 2 wk after which the continuous operation under an HRT of 1 d was initiated.

Two more PVC reactors (working volume = 2 L) were introduced at the latter part of the study. The same types of membrane modules, as described above, were used in conjunction to these reactors. One of the reactors was operated under continuous feeding mode, and the other in sequencing batch mode. Performance of these reactors was compared under two different HRTs (1 and 7 d). Under the HRTs of 1 and 7 d, both of these small reactors were operated for 2 and 6 wk, respectively. Due to the small volume of these reactors and the available large surface area of the membrane, the calculated average permeate flux was rather low. However, due to the typical operation mode (draw/fill/react) of the sequencing batch reactor, the actual (instantaneous) applied flux in case of this reactor under the HRT of 1 d was the highest of those used in this study. Further details on this aspect have been provided at the relevant sections.

2.3 Batch test description

The fungal biomass utilized for pure culture decoloration test was first grown by aseptically inoculating four pieces (1 cm^2) cut from the actively growing culture on a Potato-Sucrose-Agar plate in 200 mL colorless, autoclaved growth media (Sec. 2.1) within a 300 mL flask. The flasks were then incubated at the optimum growth temperature of 28 °C on a shaker (BR-300LF, Taitec reciprocal bio-shaker, Japan) at 80 rpm. The inoculated agar pieces grew into oval-shaped agglomerate and started to exhibit extracellular enzymatic activity (Laccase) within a few days. The thus obtained fungal biomass was harvested after 2 wk for *in vivo* test, while the filtered (0.45 μm cellulose acetate filter, Advantec, Japan) extracellular suspension was utilized as crude enzyme solution for *in vitro* decoloration test. 0.05 g (dry wt.) active fungal biomass was aseptically inoculated in autoclaved culture media including 100 mg L^{-1} of Orange II dye. In order to ascertain the contribution of biosorption to total decoloration, flasks containing same amount of fungal biomass, inactivated by autoclaving (121° C, 0.2 MPa, 15 min), were also prepared. The flasks were then incubated as descried above. Same amount of sludge from the MBR was incubated under identical conditions to assess the activity of the reactor sludge. For *in vitro* enzymatic degradation test,

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5 mL dye solution was aseptically incubated with previously harvested 5 mL crude enzyme solution (final mixture activity = 5.2 μM min^{-1}) in a 50 mL tube and then subjected to reciprocal shaking (80 rpm). Samples (0.5 mL) were taken at the specific intervals of time, diluted four times with Milli-Q water and the absorbance at the peak wavelength of the dye was measured to ascertain the extent of media decoloration.

2.4 Analytical methods

TOC was measured with a TOC analyzer (TOC-V, Shimadzu, Japan). Color measurements were carried out using a spectrophotometer (U-2010, Hitachi, Japan). Fungal enzymatic (Laccase) activity was measured by monitoring the OD468-change due to the oxidation of 2,6-dimethoxyphenol at room temperature over 2 min. Enzymatic activity was calculated from the molar extinction coefficient ε = 49.6 mM^{-1} cm^{-1} (Nyanhongo et al., 2002) and expressed in μM substrate min^{-1} (Detection limit = 0.0045±0.0007). The mixed liquor suspended solids (MLSS) concentration was estimated following the standard method (APHA, 1998). Microscopic observations (BH2 Microscope system; Olympus, Japan) were conducted to detect bacterial contamination in the fungi reactor. 1000 times magnified microscopic photograph under bright field mode was taken periodically. Transmembrane pressure (TMP), as an indicator of membrane fouling, was continuously monitored using a vacuum pressure gauge (GC 61, Nagano Keiki Co. Ltd., Japan).

3. Results and discussion

3.1 Fungal decoloration under sterile environment

3.1.1 In-vivo degradation

Excellent decoloration of Orange II dye was exhibited by the pure fungus culture (Fig. 2a). Virtually complete decoloration of the dye solution was achieved in 4 d. Extracellular enzymatic activity was detected in this culture right from the next day of inoculation. Comparison of decoloration of media containing active and inactivated fungi provided idea about the extent of biosorption of the dye. Only 1% decoloration of the media containing Orange II was observed in the flasks containing inactivated fungi, indicating a low level of biosorption of this dye. The biomass in the flask containing active fungi grew to 0.08 g from the initial 0.05 g within 7 d, but biosorption on the slight additional biomass can not account for the remarkable decoloration in this case. It is, therefore, evident that the main mechanism of decoloration was biodegradation. This observation is in line with majority of other studies employing *C. versicolor* (Wesenberg et al., 2003).

3.1.2 In-vitro degradation

Depending on the type of dye, culture media and the fungus strain under consideration, contradictory reports on the capacity of crude extracellular suspension to decolorize dye solution exist in literature (Blanquez et al., 2008; Michniewicz et al., 2008; Svobodova et al., 2008). In our study, about 55% of Orange II was degraded from a 100 mg L^{-1} solution within 1 d, beyond which the dye degradation virtually ceased (Fig. 2b). The enzymatic activity of the *in-vitro* test solution decreased by 60% in 1 d. Different factors may be responsible for enzyme deactivation (Wesenberg et al., 2003; Svobodova et al., 2008). The probable impact of enzyme deactivation on dye degradation rate, although intriguing, is beyond the scope of this study.

The levels of enzymatic activity were significantly different in the *in-vivo* and *in-vitro* test solutions. Besides, not only extracellular but also mycelium-associated enzymes are

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involved in case of *in-vivo* degradation. Therefore, direct comparison of the rate of degradation in pure culture and *in-vitro* tests would not be meaningful. Nevertheless, more relevant to our study, the involvement of extracellular enzyme in degradation of the selected dye was confirmed in the *in-vitro* decoloration test.

3.2 Performance of continuous MBR under non-sterile environment

Preliminary batch tests confirmed the significant dye degradation capacity of the pure fungus culture. However, as mentioned before, the decoloration performance of fungi in the reactors under non-sterile environment is usually significantly worse than that observed in flask cultures, particularly in the continuous flow reactors (Moreira et al., 1998; Zhang et al., 1999; Zhang and Yu, 2000; Libra et al., 2003). We, hence, observed the performance of the fungal MBR fed with a synthetic wastewater containing Orange II dye.

3.2.1 Bacterial contamination in MBR

The MBR was initially inoculated with pure fungi culture; however, it was operated without any special control of bacterial intrusion such as sterilization of feed wastewater or air. Accordingly, bacterial contamination occurred during the start-up period (Fig. 3a). However, size-based approximate fractionation of the MLSS (Jasti et al., 2006) indicated a relative abundance of fungi during continuous operation (data not shown). With the composition mentioned in Sec. 2.1, the pH of the wastewater stood at 4.5. Without any control, the pH in the MBR varied in the range of 5.5 to 6. This slightly acidic pH may have contributed to some extent to the observed relative abundance of fungi (Libra et al., 2003; Gao et al., 2006).

3.2.2 Decoloration

The absorbance of membrane-permeate and reactor-supernatant over the operation period has been plotted in Fig. 3b. Contrary to the excellent decoloration of orange II in batch test, an average overall removal (bioreactor + membrane) of 93% was observed in the MBR. The average supernatant quality indicated 82% removal by biological activity itself. In our previous study (Hai et al., 2006), over 99% removal of a polymeric azo dye (Poly S119) was consistently achieved in the continuous MBR (Table 1). However, unlike the present dye, Poly S119 showed strong adsorption on biomass, which led to its subsequent excellent retention by the cake layer on the membrane. This interpretation is supported by the fact that, for Poly S119, the permeate quality (> 99% removal) was much improved over the reactor-supernatant (68% removal) quality. On the other hand, in this study, as the Orange II dye showed lower sorption on biomass (Sec. 3.1.1), the cake layer on the membrane could not retain this dye as effectively as the Poly S119 dye. Accordingly lower overall removal of Orange II dye was achieved under the applied HRT.

Interestingly, observations following an incidence of failure of the pump supplying concentrated media to the reactor (see Sec. 2.2), which caused dilution of the reactor-media with tap water for a day or so, provided important clues to enhancement of fungal degradation. The reactor was unattended from the night of day 62, and by the time when the accident was noticed on the morning of day 64, the reactor media had already turned virtually colorless, and a large clump of fungal mass was floating on the reactor-surface. Presumably, in response to the acute starvation, the fungi agglomerated together and due to the presence of the membrane this agglomerated mass was not washed out of the reactor. As the pump was fixed and regular operation was resumed leaving the agglomerated mass as it was,

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membrane-permeate gradually turned colored in the following days (day 65-68, Fig. 3b). However, it did not deteriorate to the level that was maintained up to day 62. This level of decoloration (91 and 97% in supernatant and permeate, respectively) persisted up to day 75. Following that, the decoloration rate plummeted gradually as the agglomerated biomass progressively disintegrated. This unique observation suggested that maintenance of agglomerated morphology in contrast to the disintegrated morphology (usually observed in vigorously mixed reactors) may be one way to sustain stable fungal activity despite of the inevitable bacterial contamination.

3.2.3 Extracellular enzymatic activity

Unlike in pure culture batch test, fungal extracellular enzyme was initially of undetectable level in the MBR (Fig. 3b). Janshekar and Fiechter (1988) reported good decoloration in a stirred batch reactor under undetectable enzymatic activity. Apparently, the activity of the fungi in MBR was just enough to decolorize, but not strong enough to show extracellular enzymatic activity.

It is important to point out here that, slight extracellular enzymatic activity (as opposed to none before) was detected during the aforementioned (Sec. 3.2.2) incidence of temporary improvement in decoloration. Trace amount of activity was also detected in the MBR-permeate during this time, suggesting enzyme washout from reactor. Nevertheless, as the agglomerated biomass progressively disintegrated, the decoloration rate plummeted gradually and the enzymatic activity also diminished. Carbon or nitrogen limitation has been found essential for extracellular enzyme secretion by certain fungal species (Wesenberg et al., 2003). However, it was confirmed that our collected strain secretes enzyme in nutrient-sufficient media. Therefore, nutrient-limitation may have triggered agglomeration, but the improved enzymatic activity and consequently the improved decoloration during that incidence were not directly due to the prevailing nutrient-limitation.

Blanquez et al. (2008) reported that high enzymatic activity is not required for high level of decoloration. In that sense, transition of extracellular enzymatic activity from a moderate to substantial level may not bring about any significant change in total decoloration. However, in our study, self-agglomeration led to improvement of the enzymatic activity from undetectable to moderate level and this was accompanied by improved decoloration. This observation indicated close relationships among the factors like fungal morphology, enzymatic activity and decoloration activity under non-sterile environment.

3.2.4 TOC removal

Although white-rot fungus can initiate degradation of wide varieties of recalcitrant compounds, it has limited carbon and nutrient removal capacity as compared to bacteria (Coulibaly et al., 2003). However, consistently over 98% TOC removal (Table 1) was achieved in this study. This may be attributed to the bacterial contamination in the fungal reactor. In this study, in accordance with real textile effluent, the contribution of the dye to total TOC was rather low (5%). Accordingly, the dye removal, which varied from 93 to 97%, did not affect the TOC removal.

3.2.5 Hydraulic performance of the membrane

Since a membrane bioreactor was used in this study, routine monitoring of the TMP was conducted to confirm that membrane fouling did not influence any of the observations.

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The TMP slightly varied around 3 kPa during long-term operation, in the course of which the MLSS concentration (without any withdrawal of sludge) increased from around 5 to 17.5 g L^{-1} (data not shown). It is evident that under the cleaning strategies, the utilized module, which we developed previously (Hai et al., 2008), effectively resisted fatal fouling despite the high MLSS concentration.

3.3 Effect of bacterial contamination and fungal morphology

Incomplete removal of the dye and undetectable enzymatic activity in MBR alluded to the effect of bacterial contamination. However, to clearly manifest the effect of bacterial contamination on fungal activity, the activity of the MBR-sludge was compared to that of the pure fungus culture in batch test. Experiments were also carried out to ascertain the influence of fungal morphology on the extent of bacterial disruption of fungal activity. Such information is absent in the available literature.

3.3.1 Activity of MBR sludge

Comparison of activity of pure fungus culture and the mixed-microbial MBR-sludge in batch tests revealed moderate decoloration, no (undetectable) enzymatic activity and much faster rate of TOC consumption (Table 2) by the MBR-sludge, clearly indicating bacterial influence on fungal activity. It is worth mentioning here that the morphologies of the aforementioned two types of cultures were completely different (Fig. 4a)—while the pure culture grew in large, agglomerated oval masses, the size of the MBR-sludge was much smaller, presumably due to prolonged exposure to intense aeration within the reactor (Moreira et al., 2003) and bacterial disintegration of fungal mass (Libra et al., 2003).

3.3.2 Effect of fungal morphology

In MBR, temporary agglomeration of fungi was triggered by accidental insufficient feeding (Sec. 3.2.2). Similar to that observation, under prolonged incubation in flask, the MBR-sludge started to agglomerate, eventually forming approximately 0.5 cm granules (Fig. 4a). Furthermore, this sludge demonstrated much-improved decoloration in serial subcultivation with fresh media, substantiating the role of fungi morphology in decoloration activity in presence of bacterial contamination (Fig. 4b). No other study has reported such a phenomenon. However, still enzyme could not be detected in the media. It is likely that due to granulation the fungal activity improved to a level as to yield significant decoloration; however, in presence of bacterial disruption, it was still not strong enough to exhibit extracellular enzymatic activity.

3.4 Effect of fungal enzyme washout from continuous reactor

As has been already shown, bacterial presence significantly affects fungal activity. Wash-out of fungal enzyme (which is necessary for dye degradation) from continuous reactors is likely to exacerbate the situation. However, scarce information is available in literature regarding this aspect. In order to elucidate the probable effect of enzyme washout on decoloration activity, detailed experiments were carried out.

3.4.1 Occurrence of enzyme washout

Enzyme washout from continuous reactors is inevitable, unless some special measures are adopted to prevent that. The molecular weight of Laccase has been reported to be in the range of 59-110 kDa (Wesenberg et al., 2003), which is much smaller than the pore size of the microfiltration membrane utilized in this study. Accordingly, enzyme washout was

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expected. However, for most part of the MBR operation, enzymatic activity was undetectable in reactor-supernatant as well as in the membrane-permeate (Fig. 3b). Accordingly, occurrence of enzyme washout could not be directly proved at that part of the study. However, slight extracellular enzymatic activity was detected within MBR during the incidence of temporary self-agglomeration of fungi (day 64-75). Trace amount of activity was also detected in membrane-permeate during this time, which substantiated the occurrence of enzyme washout from the MBR beyond doubt.

3.4.2 HRT and enzyme washout

The extent of detrimental effect of enzyme-washout may depend on applied HRT. In order to investigate about this factor, one small reactor (2 L) was inoculated with the granulated sludge from the aforementioned batch test (Sec. 3.3.2). It was operated as an MBR first under an HRT of 7 d. In this case, as compared to the larger reactor operated under an HRT of 1 d, significant improvement, from 93 to 98% (Table 3), of Acid Orange II decoloration was observed. In addition, low enzymatic activity (as opposed to none before) was detected in the reactor with HRT of 7 d. Enzyme washout, as evident by its occasional detection in trace amounts in permeate, continued to occur; however, owing to the longer HRT the reactor sustained excellent decoloration, and enzymatic activity was of detectable level within the bioreactor.

3.4.3 Detrimental effect of enzyme washout

Having confirmed the occurrence of enzyme washout and its HRT-specific influence on removal performance, investigations were carried out to provide further evidence of the detrimental effect of enzyme washout on fungal activity.

Compared to the continuous process, the unique feature of sequencing batch reactor (SBR) is its cyclic operation, which necessitates withdrawal of a large amount of media at a time, and in that case application of a higher withdrawal rate is inevitable (Wang et al., 2006). If enzyme washout significantly hampers decoloration activity, then the performance would be worse in SBR, as the risk of enzyme washout through the membrane is higher from this reactor due to higher instantaneous withdrawal rate. To test this hypothesis, the performance of a continuous and sequencing batch MBR (Reactor # 2, 3 in Table 3, respectively) was compared under same HRTs.

Under an HRT of 7 d, the sequencing batch MBR, in fact, exhibited slightly better or comparable decoloration, enzymatic activity (Table 3) and better granulation (not shown). Aerobic granulation technology has been extensively reported in SBRs (Wang et al., 2006). The characteristic cyclic abundance and scarcity of substrate during SBR operation may have triggered better granulation and higher enzymatic activity. It is also important to mention that although the instantaneous flux for the SBR reactor (0.02 m^3 m^{-2} d^{-1}) was higher than the continuous reactor (0.0053 m^3 m^{-2} d^{-1}), owing to the small volume of the reactor and the available large surface area of the membrane, it was still comparable to that applied for the larger reactor (#1 in Table 3) operated under continuous mode. Apparently, under the applied flux, the extent of enzyme washout from the sequencing batch MBR was not critical enough to offset the advantage induced by better granulation.

Nevertheless, the detrimental effect of enzyme washout was proved beyond doubt when both these reactors were operated under an HRT of 1 d. The decoloration efficiency of

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both the reactors (especially that of the sequencing batch MBR) plummeted significantly (Table 3). The reactor-sludge under the shorter HRT appeared more disintegrated (not shown) and enzymatic activity was no more detectable in any of the reactors. The drastically deteriorated performance of the sequencing batch MBR under the shortened HRT may be attributed to higher enzyme-leakage through the microfiltration membrane from this reactor under the corresponding higher instantaneous withdrawal rate (in comparison to continuous MBR).

3.5 Interrelations of the factors

In this study, unlike in pure culture batch test, the removal of the dye within MBR was incomplete and the enzymatic activity was undetectable (Fig. 3b, Sec. 3.2). Separate batch tests confirmed the low decoloration and undetectable enzymatic activity of the bacteria-contaminated sludge in MBR, substantiating detrimental effect of bacterial contamination on fungal activity (Table 2, Sec. 3.3.1). It is likely that fungal enzymatic activity or the secreted enzyme itself was significantly destabilized by bacteria (Libra et al., 2003). However, independent observations in the MBR (Day 64-75, Fig. 3b) and parallel batch studies (Fig. 4b, Sec. 3.3.2) revealed improved enzymatic and decoloration activity of agglomerated sludge. These observations indicated close relationships among fungal morphology, enzymatic activity and decoloration activity under non-sterile environment. In addition, this study demonstrated the occurrence of enzyme washout and its HRT-specific influence on removal performance (Table 3, Sec. 3.4). During that investigation, better decoloration and enzymatic activity as well as better granulation was noticed under longer HRT (lower level of enzyme washout). Taking all these novel observations into account, it can be stated that these factors (e.g., bacterial disruption, fungal morphology, enzyme washout) are interrelated. A schematic in the supplementary data section depicts the studied interrelated key factors governing fungal activity in a continuous reactor under non-sterile environment.

3.6 Insight into ways to enhance performance

We previously demonstrated (Hai et al., 2006) in case of a dye with high biosorption that de-coupling of the dye retention time and HRT is possible in a membrane-coupled fungi reactor, where, owing to biosorption, the dye was retained by the cake-layer on the membrane, and was subsequently degraded by fungi. Accordingly, satisfactory removal even under low titer of fungal activity in the presence of bacterial contamination was achieved. In this study, however, the removal of the dye with very low biosorption was incomplete in the MBR. Such dye-specific removal performance of the MBR (Table 1) obviously necessitates modification of the proposed process. Observations made in this study concerning the factors governing the decoloration activity provide useful clues in this regard.

Our results (Fig. 3b, day 64-75; 4b) suggest that maintenance of agglomerated morphology may be one way to sustain stable fungal activity despite of the inevitable bacterial contamination. We observed spontaneous agglomeration in case of nutrient limitation. The combined strategies of nutrient-limited condition and partial biomass renovation seem useful in this respect (Blanquez et al., 2008). However, a simpler and, perhaps, more sustainable option would be to maintain agglomerated growth on immobilizing supports (e.g., metal or plastic mesh) placed in a compartment within the MBR. It is worth-mentioning that sterile investigations usually report better activity of immobilized cultures (Ramsay et al., 2005).

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Another important issue is the washout of fungal enzyme. Minimization of enzyme washout from continuous reactors may do away with the requirement of long HRT. Activated carbon which can adsorb varieties of organics including dye (Hai et al., 2007) can be useful in this sense. Zhang and Yu (2000) previously reported improved decolorization in batch tests due to adsorption and close contact of dye as well as extracellular enzyme on activated carbon. Both elongation of the retention time of dye and minimization of enzyme washout may be achieved by application of simultaneous adsorption within MBR. Preliminary results from our ongoing investigation support the suitability of the proposed modifications.

4. Conclusions

In this study, through a set of novel observations, the role of factors like bacterial disruption, fungal morphology and enzyme washout in a continuous fungi reactor was clearly demonstrated. In the light of that, certain means to enhance decoloration were proposed.

In contrast to virtually complete decoloration of an azo dye (Acid Orange II) in pure culture batch tests, the MBR achieved 93% removal during non-sterile operation at an HRT of 1 d. Long-term observation of the performance of MBRs under different HRT and feeding mode (continuous or fed-batch) as well as batch tests with pure fungus culture and reactor-sludge enabled identification of the reasons for the incomplete removal. Results from this study demonstrated detrimental effect of bacterial contamination on fungal activity and suggested close relationships among fungal morphology, enzymatic activity and decoloration activity under non-sterile environment. This study also demonstrated the occurrence of enzyme washout and its HRT-specific detrimental influence on removal performance. Maintenance of agglomerated morphology and simultaneous activated carbon adsorption within MBR were proposed to improve the MBR performance.

Acknowledgments

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FIGURES

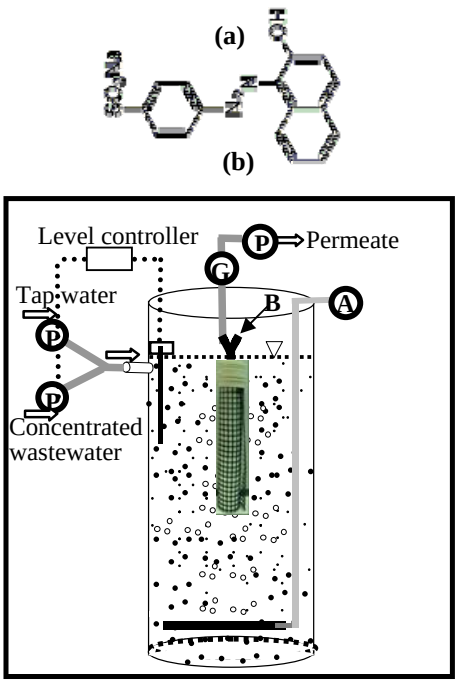

Fig. 1 (a) Structure of Acid Orange II dye (b) schematic of MBR setup
(A: Air pump, B: Backwash, G: Vacuum gauge, P: Pump)

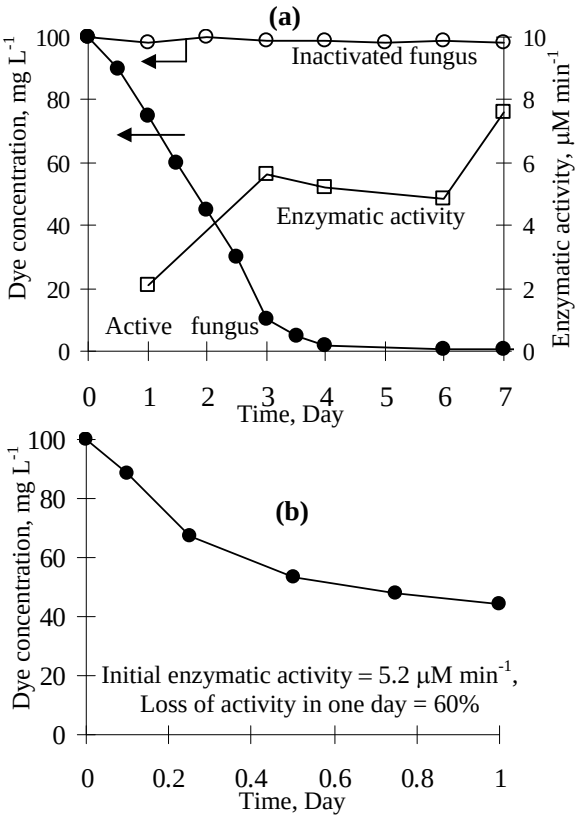

Fig. 2. Dye degradation in sterile batch tests
(a) Degradation by pure fungus culture
(b) *In-vitro* enzymatic degradation

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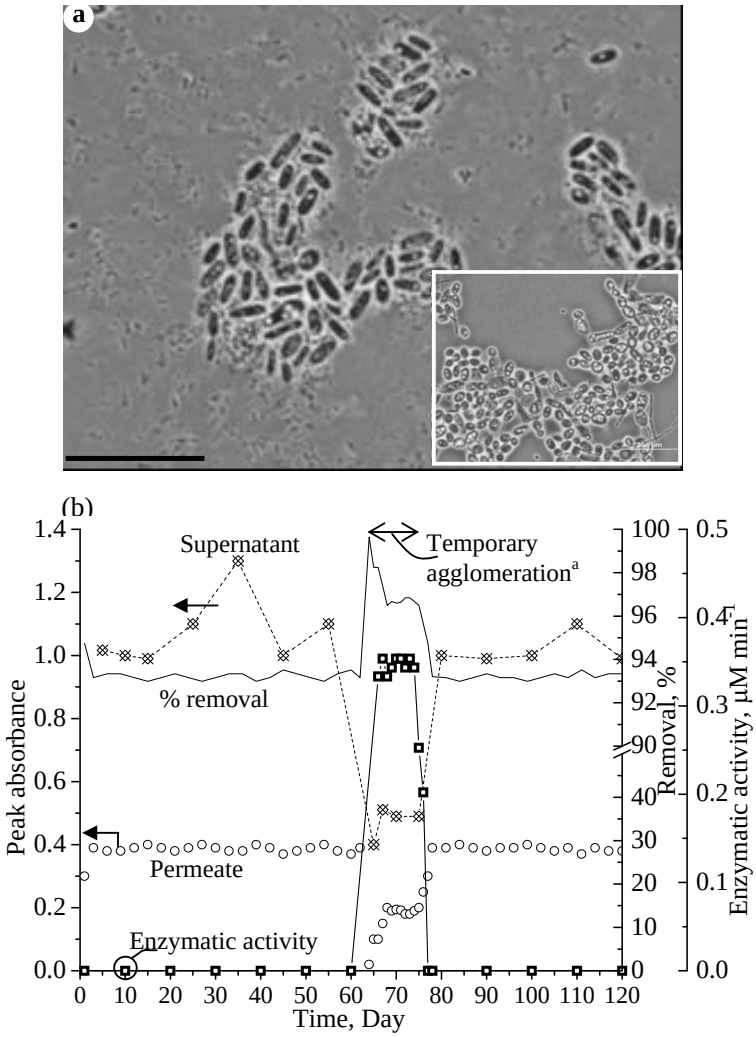

Fig. 3 (a) Microscopic observation revealing bacterial contamination in the fungal MBR (smaller dots represent bacteria, the black bar indicates 20µm; inset diagram shows pure fungus culture used for inoculation); (b) Corresponding long-term decoloration and enzymatic activity within MBR ([a]trace amount of enzymatic activity was detected occasionally in membrane permeate as well during temporary agglomeration period)

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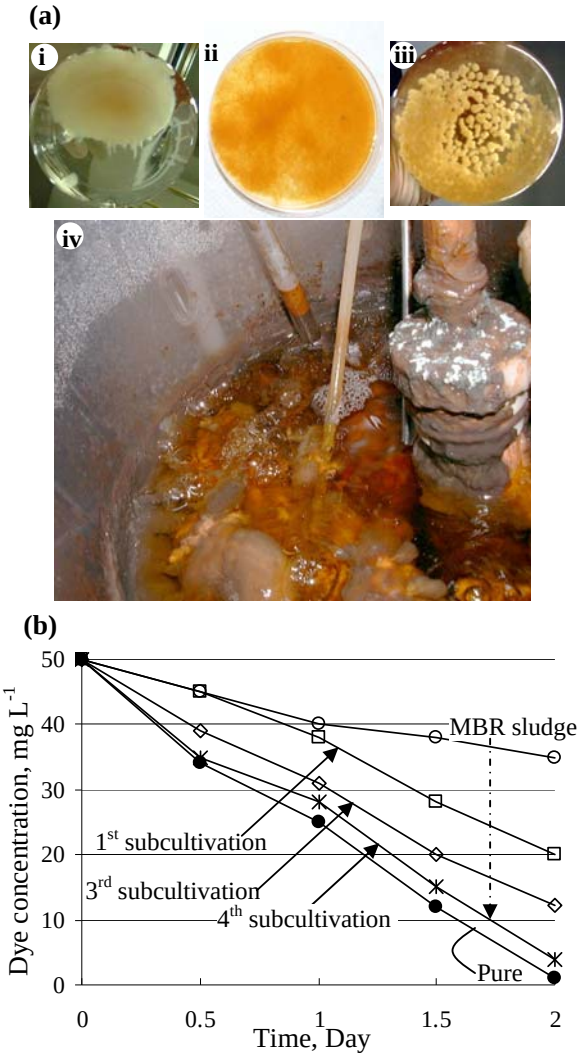

Fig. 4 **(a)** Different biomass morphologies encountered during this study; i. Pure fungus culture, ii. MBR-sludge, iii. Granulation of MBR-sludge under prolonged incubation in flask, iv. Temporary agglomeration of fungi in MBR (refer to Fig. 3b). **(b)** Improved decoloration along with progressive granulation of MBR-sludge in flask (decoloration by pure culture was plotted as a reference)

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TABLES

Table 1. Dye-specific removal performance of fungal MBR

Dye	Average removal, %			Reference
	Dye		TOC	
	Supernatant	Permeate		
Poly S119[a]	68	99	>97	Hai et al., 2006
Orange II[b]	82	93	>98	This study

[a] Polymeric azo dye (high biosorption)
[b] Low-molecular weight, mono-azo dye(low biosorption)

Table 2. Comparison of activity of pure fungus culture and MBR-sludge in batch test (7 days)

Biomass	Removal, %		Enzymatic activity, $\mu M\ min^{-1}$
	Color	TOC	
Pure fungi	> 99	18	8
Reactor sludge	48	94	0[a]

[a] undetected

Table 3. Performance of MBRs under different HRT and operation mode

Reactor no.	Operation mode	Volume (L)	HRT (d)	Membrane operation			Color removal, %	Enzymatic activity, $\mu M\ min^{-1}$
				On/Off [a] (min)	Flux ($m^3\ m^{-2}\ d^{-1}$)			
					average	Instantaneous		
1	Continuous	11.8	1	5/5	0.011	0.022	93	0 [d]
2	Continuous	2	1	6/24	0.0019	0.009	88	0 [d]
			7	3/57	0.0003	0.0053	98	0.35[e]
3	Sequencing batch	2	1	Draw/fill/react[b]	0.0019	0.07	65	0 [d]
			7	Draw/fill/react[c]	0.0003	0.02	99	0.55[e]

[a] Withdrawal and idling period, [b]Four cycles of $[Draw\ (10)/fill\ (5)/react\ (345)]_{min}$ each day, [c]One cycle of $[Draw\ (20)/fill\ (5)/react\ (1415)]_{min}$ each day, [d] undetected, [e] trace activity was occasionally noticed in the membrane-permeate as well.

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Supplementary material

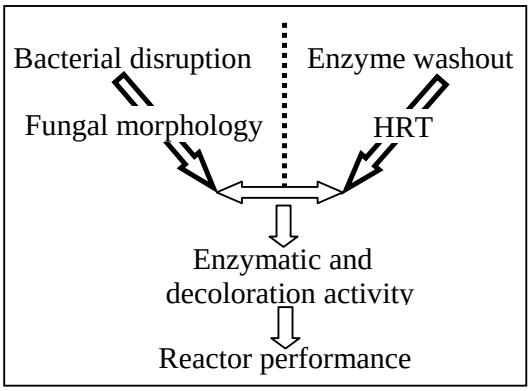

Figure. The studied interrelated factors governing performance of a fungal reactor during continuous operation under non-sterile environment

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